

# New Prospects for Glycopeptide Based Analgesia: Glycoside-Induced Penetration of the Blood-Brain Barrier

Muthu Dhanasekaran and Robin Polt\*

The Carl S. Marvel Laboratories, Department of Chemistry, The University of Arizona, Tucson, Arizona 85721, USA

**Abstract.** Antinociceptive effects and BBB transport properties of glycosylated enkephalin derivatives are reviewed. Previously, the application of enkephalins as analgesics has been retarded by their poor stability *in vivo* and by their inability to effectively penetrate the blood brain barrier. This shortcoming has been overcome by glycosylation, paradoxically leading to enhanced BBB transport *via* transcytosis. Principal design considerations required for enhanced binding, stability and transport of opioid peptides are reviewed. Modifications of the peptide backbone and side chains to achieve optimal receptor binding ( $\mu$ /-selectivity) are presented. The importance of reversible binding between the glycopeptide and membranes is emphasized, and several pertinent examples of peptide-membrane interactions are discussed in the light of glycopeptide transport and opioid binding. An “amphipathic hypothesis” is introduced as a rationale for the observed BBB penetration of the opioid glycopeptides.

**Keywords:** Glycopeptide, Opioid, Enkephalin, Blood-Brain Barrier, Analgesia, Transcytosis, Morphine.

## INTRODUCTION

The use of opiates as crude preparations for the relief of pain and the control of dysentery stretches back into prehistory. Ancient Sumerians are believed to have harvested and used opium poppies for analgesic and euphoric purposes at least as early as 3400 BCE [1], and that opiate use spread from the Sumerians to the rest of the world. The sap of the poppy (*Papaver somniferum*) was named “opium” after the Greek word for “juice”, and later was termed as a “narcotic” from the Greek word for “stupor”. Friedrich Sertürner isolated the principal alkaloid from opium in pure form in 1806, and named it morphine after Morpheus, the Greek god of dreams [2] Robiquet isolated codeine in 1832 and workers at Merck isolated papaverine in 1848. These congeners of morphine display strong analgesic and addictive properties, and have been the subject of intensive pharmacological modifications, as well as serving as important benchmarks for many structure activity relationship (SAR) studies with the morphinan ring system and related pharmacophores. Morphine is also an important benchmark for clinical studies, and is still the most widely prescribed injectable opioid today [3] despite its narcotic side effects, which include respiratory depression, [4] drowsiness, stupor, nausea, biliary pressure increases, constipation and immunosuppressive effects [5] In addition to these problems, tolerance and physical dependence can develop with repeated use. Acute opioid toxicity from overdose may result in coma and/or convulsions, and death; invariably resulting from respiratory failure.

Endogenous opioid peptides, lumped together under the generic term *endorphins*, have been the subject of fascination by the scientists since their discovery in the mid 1970's.

They have also been the subject of intense scrutiny by pharmacologists and neuroscientists [6] These are the substances produced within the human brain that relieve pain, allow one to experience “runner’s high” or to enjoy a chocolate bar. Although these experiences are subjective, there is no question that the endogenous opioid systems play critical roles in diverse sensory, emotional, motivational, and cognitive functions. If these naturally occurring opioid peptides and their derivatives could be rendered permeable to the blood-brain barrier, then a vast new vista of psychopharmacology would be opened to exploration by medical researchers. Such brain-permeable neuropeptide-based drugs might have the potential for extremely selective pharmacological intervention without the narcotic side effects associated with morphine.

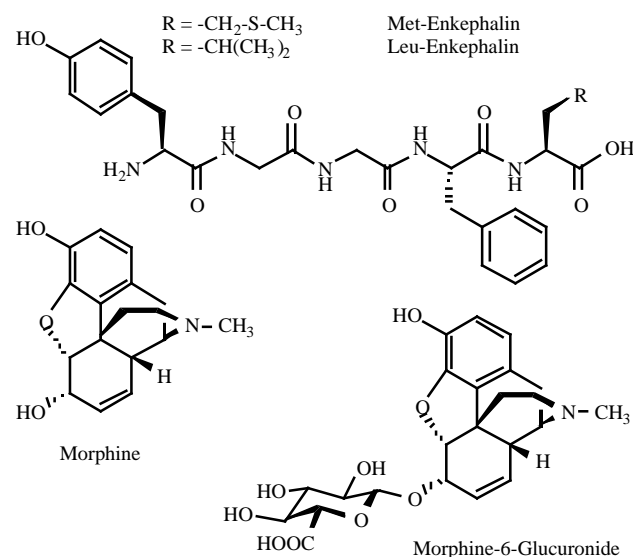


Fig. (1). Comparison of Morphine and Enkephalin Structures.

\*Address correspondence to this author at the Carl S. Marvel Laboratories, Department of Chemistry, The University of Arizona, Tucson, Arizona 85721, USA; Tel: (520) 621-6322; Fax: (520) 621-8407; Email: polt@u.arizona.edu

## RECEPTOR SUBTYPES

Simon, Snyder and Terenius simultaneously reported the discovery of the receptors responsible for binding opioid peptides in a stereoselective manner in 1973 [7]. It has been firmly established that there are at least 3 multiple opioid subtypes:  $\mu$ ,  $\kappa$ , and  $\delta$ . Existence of other receptors, such as  $\sigma$  and  $\nu$  have been suggested, but have not been confirmed [8,9]. Opioid receptors have been found throughout the mammalian CNS and peripheral nerve tissues such as the mouse vas deferens, guinea pig ileum and human gastrointestinal tract. The classical opiate morphine is a potent agonist at  $\mu$ -opioid receptors, but also produces agonism at  $\kappa$  and  $\delta$  receptors with low binding affinity. Sub classes of receptors ( $\mu_1$ ,  $\mu_2$ ,  $\mu_3$ ,  $\mu_4$ ,  $\mu_5$ ) have also been proposed based on their differing pharmacology, [10] or based on their differing location ( $\mu_1$ ,  $\mu_2$ ).

Except for the putative  $\nu$ - and  $\sigma$ -receptors, all the major opioid receptor subtypes have been sequenced in both mice and humans, and were cloned by the year 1994. The first successful cloning of the  $\delta$ -receptor was reported by Kieffer *et al.* and Evans *et al.* in 1992 [11]. The  $\mu$ -receptor was cloned in 1993 by Fukuda *et al.* and by Chen *et al.*; [12] the  $\kappa$ -receptor was cloned by Yasuda *et al.* and Minami *et al.*, also in 1993 [13]. The situation is complicated by at least seven different splice variants of the MOR-1 gene, [14] and probably among the other subtypes as well. The widespread use of knock-out mice has added a new dimension to the study of receptors *in vivo* [15]. Likewise, homo- and hetero-oligomerization of receptors remains an area of active research [16].

The glycosylation state of the native opioid receptors has not been extensively studied. The  $\mu$ ,  $\kappa$ , and  $\delta$ -receptor subtypes share both sequence and structural features, and are integral membrane proteins. The opioid receptors are G-protein coupled receptors, and all are believed to possess seven membrane-spanning  $\alpha$ -helical segments. Most of the homology between the  $\mu$ ,  $\kappa$ , and  $\delta$ -receptors lies in the highly helical membrane-spanning domains. The differences that largely seem to define these receptors are in the second and third extra-cellular loops, which are believed to be associated with ligand specificity, [17] as well as in the intracellular carboxy termini and the extracellular amino termini. The binding sequence Tyr-Gly-Gly-Phe located at the N terminal of the endogenous opioid peptides (message sequence) is highly conserved, which suggests that the binding site is similar in all of the receptors. The endomorphins are an exception to this rule, and the precursor peptides for the endomorphins are not yet known, although the relevant nucleotide sequences appear hundreds of times within the human genome. There is still a paucity of detailed structural knowledge about the precise binding site of the opioid receptors despite decades of SAR work, although many proposals have been put forth. Even less is known of the receptor and ligand structural features that distinguish agonism from antagonism. The lack of molecular details makes the successful design of ligands specific for the receptors a challenge, and this process remains largely empirical.

## ENDOGENOUS OPIOID PEPTIDES

The pharmacology of opiates was greatly stimulated by Kosterlitz's discovery of two endogenous pentapeptides Met-enkephalin and Leu-enkephalin in 1975 [18]. Other endogenous opioid peptides were discovered in the mammalian brain in the years following, and at present dozens of endogenous opioid peptides have been characterized. Initially, there was a great deal of enthusiasm in pharma for the use of enkephalins and other opioid peptides from the brain as analgesics, however this enthusiasm was dampened considerably by the determination that these compounds did not penetrate the blood-brain barrier (BBB), and had very short half-lives in the blood stream. Extensive SAR studies in many laboratories have resulted in potent and selective opioid agonists and antagonists, [19] and a few of these have showed greatly increased half-lives in serum [20]. Penetration of the BBB by peptides remains enigmatic, however, despite the application of a great deal of effort, recently driven by re-invigorated efforts to apply the fruits of biotechnology to clinical problems [21].

Endorphin is a generic term for the 3 families of endogenous neuropeptides, the enkephalins, the dynorphins and the  $\delta$ -endorphins [22]. The prototype,  $\delta$ -endorphin was first isolated in 1976. Although  $\delta$ -endorphin (31 residues) contains the Met-enkephalin peptide sequence on the N terminus,  $\delta$ -endorphin and its kin are produced from proopiomelanocortin (POMC), whereas the enkephalins arise from a different precursor protein, proenkephalin [23]. Likewise, the dynorphins are derived from prodynorphin, which yields more than seven peptides, including Leu-enkephalin. Since opioid and non-opioid peptides (*e.g.*  $\delta$ -MSH, ACTH,  $\delta$ -LPH) are enzymatically cleaved by various proteases from the much longer peptide, the cleavage event can result in a diverse set of peptides of varying length, allowing for modes of biological regulation that are much more sophisticated than simple up-regulation or down-regulation of the genetic expression levels.

A fourth "orphan" receptor, the opioid-like receptor (ORL), has been identified and cloned, [24] and may be regarded as a member of the opioid receptor family. Its endogenous ligand appears to be nociceptin, or orphanin-FQ, which differs from dynorphin in that it bears F instead of Y at the N-terminus. Nociceptin, like the opioid peptides, is derived from a larger precursor protein, proorphanin [25]. Unlike the opioid receptors, this receptor seems to *raise* the threshold for pain, rather than decreasing pain, and its inclusion as an opioid receptor is debatable. This ligand has little or no binding affinity for the classical opioid receptors.

The largest known member (25 residues) of the proenkephalin opioid peptides is adrenal peptide E, which is reported to be a potent  $\mu$  and  $\delta$  agonist [26]. Peptide E has a Met-enkephalin sequence on the N terminus and a Leu-enkephalin sequence embedded at the C terminus. The peptide  $\delta$ -endorphin was shown to promote analgesia similar to morphine, and is a very potent  $\mu$  agonist [27]. Endomorphins I and II are believed to be the endogenous ligands for the  $\mu$  receptor, but the precursor proteins have not

**Table 1. Naturally Occurring Opioid Peptide Sequences**

Peptide	Sequence	Subtype
Met-Enkephalin	YGGFM	$\mu$ /
Leu-Enkephalin	YGGFL	/ $\mu$
Dynorphin A	<u>YGGFLRRIRPKLKWNNQ</u>	( $\mu$ )
Dynorphin B	<u>YGGFLRRQFKVVT</u>	( $\mu$ , )
-Neoendorphin	<u>YGGFLRKY</u>	( $\mu$ , )
-Neoendorphin	<u>YGGFLRKYP</u>	( $\mu$ , )
$\delta$ -Endorphin	<u>YGGFMTSEKSQTPLVTLFKNAIKNAYKKGE</u>	$\mu$ /
Peptide E	<u>YGGFMRRVGRPEWWMQDYQKRYGGFL</u>	$\mu$ /
Peptide F	GGEVLGKRYGGFM	—
Nociceptin	FGGFLRRIRPKLKWNNQ	ORL
Deltorphin	<u>YmFHLMD-CONH<sub>2</sub></u>	
Dermorphins	<u>YaFGYPS-CONH<sub>2</sub></u>	$\mu$
Morphiceptin	<u>YPFP-CONH<sub>2</sub></u>	$\mu$
-Casomorphin	<u>YPFPGPI</u>	$\mu$
Endomorphin-1	<u>YPWF-CONH<sub>2</sub></u>	$\mu$
Endomorphin-2	<u>YPFF-CONH<sub>2</sub></u>	$\mu$
Rubiscolin-6	<u>YPLDLF</u>	

yet been identified [28]. The agonist dynorphin-A (1-17), which contains the Leu-enkephalin sequence at the N-terminus, was isolated by Goldstein *et al.* in 1979 [29]. More recently a family of opioid peptides lacking the prominent YGGF “message segment” common to the mammalian opioid peptides was shown to be present in the skin of the frog *Phyllomedusa bicolor*. The presence of the enantiomeric D-amino acids in the amphibian peptides makes the  $\mu$ -selective dermorphins and  $\delta$ -selective deltorphins unusual, at least in a mammalian context. Additionally, the YxF “message segment” can replace YGGF [30]. Pharmacologically active opioid peptides that contain a similar YPX “message segment” have also been obtained from plant sources, where X is usually an aromatic amino acid (*e.g.* -casomorphin, endomorphin-2, *inter alia*), [31] or less commonly, an aliphatic amino acid (*e.g.* the rubiscolins). These latter peptides have been observed in intestinal enzymatic digests, and although their biological significance is not clear, they appear to have weak oral antinociceptive activity in the mouse tail pinch assay [32].

### INCREASING PEPTIDE STABILITY

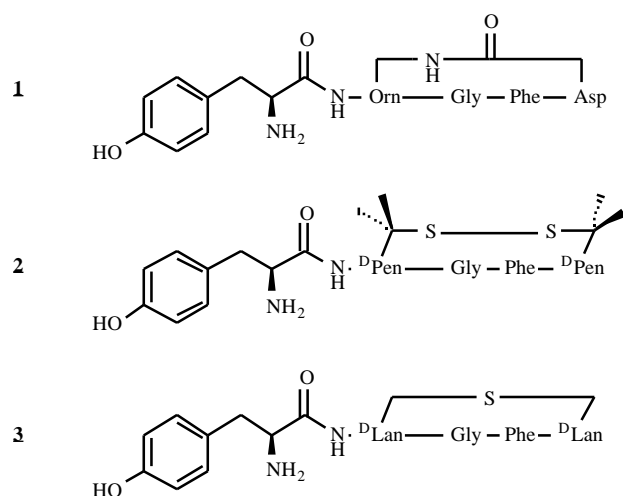
Endogenous peptides generally have a short lifetime *in vivo* due to enzymatic biodegradation, and consequently have poor bioavailability in tissues and organs. Therefore, it has been a challenge to improve the stability of an enkephalin while still maintaining its potency and selectivity [33]. Many enkephalin analogs are now available that are

quite stable to enzymes encountered *in vivo*. Several strategies have been successful to increase enzymatic stability [34]. In fact, many of the same chemical modifications used to increase potency and selectivity of opioid peptides (*e.g.* non-natural amino acids, cyclization, and chirality inversion, *vide infra*) also render the resulting peptide inert to peptidases. However, almost invariably they do not cross the blood brain barrier to an appreciable extent.

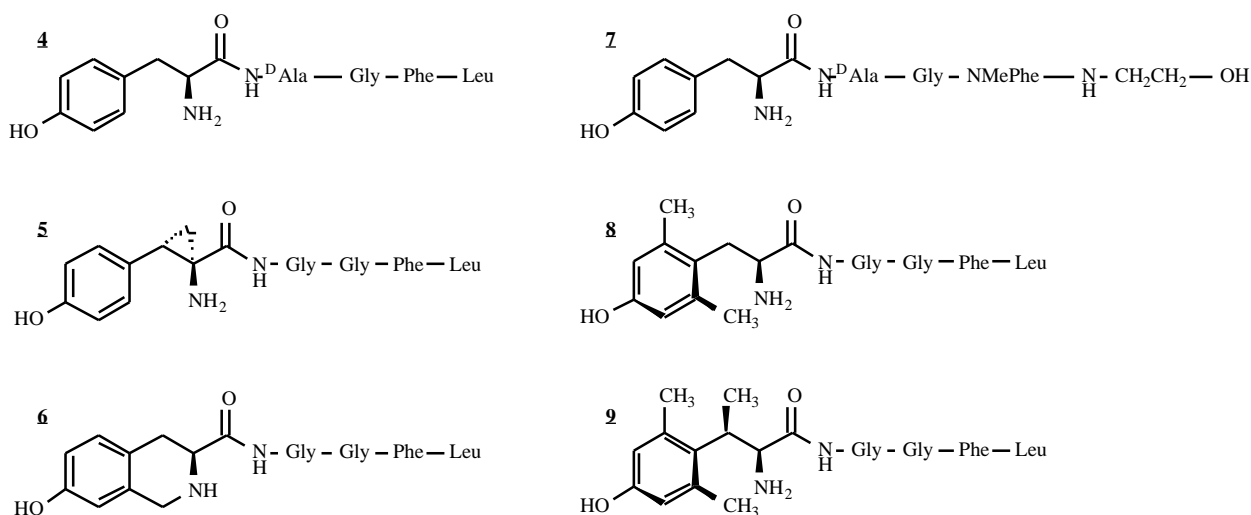
### PEPTIDE-BASED ANALGESIA

Attempts to develop enkephalin-based pharmaceuticals began immediately after their discovery. Early work suggested that a Tyr-Xxx-Gly-Phe motif was required for opioid agonism, and that enhanced potency and enzymatic stability could be observed when Xxx was a D-amino acid. It was observed that even greater stability and receptor selectivity could be achieved by introducing cyclic constraints [Fig. (2)] into opioid peptides by embedding lactam bridges (Orn<sup>2</sup>—Asp<sup>4</sup>, **1**), [35] disulfide bonds (D-Pen<sup>2</sup>—D-Pen<sup>5</sup>, **2**), [36] or sulfide linkages (lanthionine, **3**) [37] into the message sequence. Improvements have also been made by controlling side-chain orientation, or  $\beta$ -space, [38] by the use of unnatural amino acids [Fig. (3)], such as D-amino acids (**4**, **7**), [39] cyclopropyl amino acids (**5**), [40] cyclic amino acids (**6**), [41] and amino acids bearing additional alkyl groups (**7**, **8**, **9**) [42].

Taken as a whole, this work represents a remarkable achievement by the opioid peptide community, which can



**Fig. (2).** Cyclic Enkephalin Analogs.



**Fig. (3).** Acyclic Enkephalin Analogs with Modified Amino Acids.

boast of extremely potent opioid peptide tools with exquisite selectivity. These tools have been used, and are still being used, to pose and to answer a number of important questions of interest to a diverse set of life scientists. Unfortunately, despite their potent binding, selectivity, and enormous utility as pharmacological probes, none of these opioid agonists effectively penetrate the blood-brain barrier, and thus do not produce analgesia in a therapeutically useful manner.

### THE BLOOD-BRAIN BARRIER (BBB)

One of the remaining problems preventing the use of opioid peptides as drugs is poor bioavailability, which is due to the low penetration across various biological barriers, especially cellular membranes [43]. The BBB acts as a barrier to unnecessary substances, and admits vital nutrients for proper function of the CNS [44]. The flow is bi-directional, allowing the export of materials from the CNS (efflux transport) and the import of materials from the blood (influx transport). Ehrlich was among the first to observe the

blood-brain barrier in the late 19<sup>th</sup> century when he examined the effect of the dye, trypan blue, on animals after *i.v.* administration. Upon dissection, he found that the brain remained a grayish-white color while all other tissues were stained blue. He reasoned that the brain and the surrounding cerebrospinal fluid (CSF) must be protected from the dye, yet at the same time the BBB must allow the passage of essential nutrients, hormones, and metabolites to the brain. Since then, it has been found that the tight junctions of the endothelial cells in brain capillaries are responsible for the BBB.

The BBB of the brain has two major components. The endothelial layer lies between the arterial blood in the brain capillaries and the interstitial fluid of the brain. In humans, the surface area of the endothelial layer in the brain is about 21 m<sup>2</sup> [45]. The epithelial layer lies between venous blood and the CSF in the choroid plexus, and has a total surface area of about 0.021 m<sup>2</sup> in humans [46]. At the spinal cord, the BBB only consists of the endothelial barrier. Thus, CSF

is created in the choroid plexus and flows around the brain and down to the spinal column. Complete turnover of the CSF takes about 5 hours in humans [47]. The BBB represents not only a physical obstacle, but a metabolic one as well, possessing both oxidative enzymes and peptidases such as aminopeptidase, arylamidase and enkephalinase. Thus, metabolically unstable substances (*e.g.* peptides) are rapidly degraded before they reach the CSF. This enzymatic gauntlet may be as important as the lipophilic barrier in excluding peptide pharmaceuticals from the CNS. It should also be noted that entry to the CSF does not guarantee that a drug will enter the brain, as many peptides are rapidly exported back to the bloodstream [48].

The transport of drugs across the BBB falls into two broad categories: passive diffusion across the cell membrane and passage mediated by a specific transport mechanism. Since the peptides are relatively large and hydrophilic, they do not cross BBB by passive diffusion and they require another transport mechanism. Invasive brain drug delivery strategies have been the most widely used techniques to deliver drugs behind the BBB using *i.c.v.* (intracerebroventricular) infusions, or intrathecal implants [49]. While there are medical situations where such invasive techniques are justified in humans, non-invasive methodologies offer the potential for much wider therapeutic application of analgesic drugs based on opioid peptides. While *i.c.v.* animal studies are instructive and are described in this review, it is understood that such invasive techniques are essentially neurosurgical in nature, and do not represent a satisfactory solution to the drug delivery problem.

#### TRANSPORT MECHANISMS

There are five modes of transport across the endothelial cells of the brain capillaries as illustrated in Fig. 4 [50].

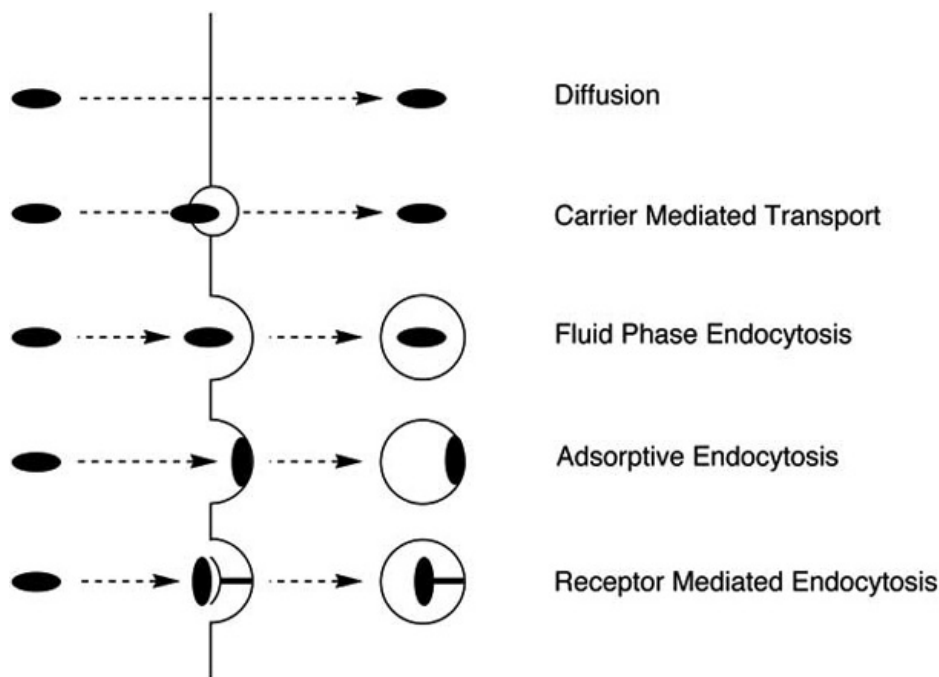


Fig. (4). Five Modes of Transport across the Blood-Brain Barrier.

Many pharmaceutical agents, such as morphine, penetrate the BBB by passive diffusion across the cell membrane. Passive diffusion of a molecule is determined by its molecular size, charge, lipophilicity and hydrogen bonding capability because the BBB generally behaves as a continuous lipid bilayer. The larger and more polar a molecule is, the lower its penetration rates into the brain and CSF by passive diffusion. Thus, based on passive diffusion alone, one would not consider that large polar molecules such as peptides would ever become useful CNS drugs. Carrier-mediated transport may be active, in which ATP is consumed upon transport, and is normally unidirectional, or may be facilitative, which is driven by a concentration gradient, and can function in either direction. Glucose, which is the major energy source for the brain, is facilitatively transported *via* the glucose transporter (GLUT-1) [51]. The amino acids are transported across BBB by various transporters [52]. There are other transporters that transport both small peptides [53] as well as proteins [54]. Molecules, including large peptides, can also cross the BBB by endocytosis. There are several types of endocytosis such as fluid-phase endocytosis (FPE), adsorptive mediated endocytosis (AME) and receptor-mediated endocytosis (RME).

#### PEPTIDE DRUG DELIVERY TO THE BRAIN

It has been shown that small, but measurable amounts of enkephalins and other small peptides are taken up by the brain *via* a saturable peptide transport mechanism [55]. Davis and co-workers have shown that the peptide DPDPE, a specific enkephalin analog, crosses BBB by a combination of diffusion and saturable mechanisms, and that biphalin ([Tyr-DAla-Gly-Phe-NH<sub>2</sub>]) does so by diffusion and *via* the large neutral amino acid carrier [56]. The peptide E-2078, an

analog of Dynorphin<sub>1-8</sub>, was shown to cross BBB via adsorptive mediated endocytosis (AME) and transcytosis [57]. Recently, it has been shown that the peptide H-Tyr-DArg-Phe-Ala-OH (TAPA), a  $\mu$  specific dermorphin analog, is transported through BBB by adsorptive-mediated endocytosis [58]. It has been found that peptide size is not as important for adsorptive transcytosis (AMT). Optimal conditions of lipophilicity and cationic charge of the peptides are important for efficient transport across BBB [59]. The growing evidence that peptides can cross BBB should encourage researchers to investigate the development of peptides as drugs for CNS diseases, particularly in cases where only picomolar concentrations of the peptide are required in the brain for therapeutic effects.

## VECTORS FOR BBB PENETRATION

In vector-based drug delivery approach [60] generally there is no need to modify the peptide sequence, rather it is conjugated to a substance, the vector, such as a lipophilic moiety, a transporter peptide sequence, or a protein that is normally transported by passive-, carrier-, adsorptive- or receptor-mediated endocytosis. In most cases however, the conjugate must then be converted to an active compound through enzymatic and/or chemical means, before or after reaching the site of action [61]. Bodor and co-workers used a novel enzyme-based transport vector strategy in which they modified both N- and C-termini of the enkephalin peptide by attaching enzymatically labile steroids. This "packaged" enkephalin crossed the BBB by passive diffusion and showed considerable antinociceptive effects, but lacked the aqueous solubility necessary for effective serum transport [62]. Very large doses (5-20 mg/kg) were required to induce centrally mediated antinociception, and the drug had to be dissolved in ethanol or dimethylsulfoxide (DMSO) prior to administration.

In another approach, Borchardt and co-workers made an attempt to improve the BBB permeation of DADLE, an enkephalin analog, by making cyclic prodrugs using an acyloxyalkoxy linker, a coumarinic acid linker and an oxymethyl-modified coumarinic acid [63]. These drugs showed higher plasma concentrations of DADLE after *i.v.* administration, compared with *i.v.* administration of DADLE alone. Though the lipophilic prodrugs possess more favorable physicochemical properties, higher intrinsic BBB permeation, improved stability *in vivo* and bioconversion to parent drug, they fail to accumulate in the brain due to efflux transporters (e.g., P-glycoprotein) [64]. Similarly, ester prodrugs of the tyrosine phenolic group of the enkephalins were synthesized. These prodrugs showed good chemical stability and were able to transport across Caco-2 cells, but BBB studies have not yet been reported [65].

## NANOPARTICLE TECHNOLOGY

Nanoparticle technology has been used to improve BBB transport. This technology has been tested with dalargin (Tyr-D-Ala-Gly-Phe-Leu-Arg), a Leu-Enkephalin analog. Dalargin adsorbed on the surface of poly(butylcyanoacrylate) nanoparticle (NP) able to induce central antinociceptive effects [66,67]. The NPs carrying the

adsorbed peptides can cross the BBB as intact molecules and act on central nervous system [68]. It has been suggested that the detergent present in the preparation of nanoparticle coated drug is responsible for the observed BBB transport of the drug by disrupting/opening the BBB endothelial cells [69]. However, for the drug delivery to occur, the peptide has to be physically associated with the nanoparticles [70]. The advantage could be low drug dose needed while maintaining its therapeutic effects. There has been no specific mechanism advanced explaining how the drug is released from the nanoparticle. Recent findings suggest that there may be no toxicity due to nanoparticles (*i.e.* there is no generalized disruption of the endothelial monolayer).

## LIPIDATION

1-Adamantane derivatives of [D-Ala<sup>2</sup>]-Leucine enkephalin (*e.g.* **10**, Fig. 5) were prepared to improve the delivery to the brain of enkephalin [71]. This analog is lower in activity when the adamantane moiety is attached to the N-terminal. The adamantyl ester showed a 100 fold increase in lipid solubility compared with the unmodified enkephalin and CNS-mediated antinociception when injected *i.v.* in high doses (5-50mg/kg).

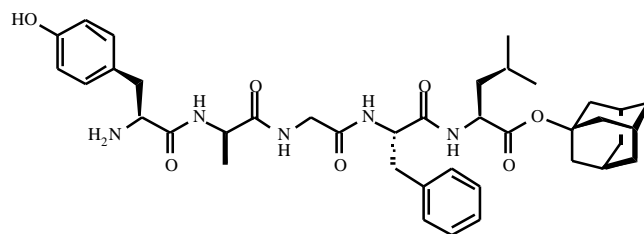


Fig. (5). 1-Adamantyl Esters of [D-Ala<sup>2</sup>]-Leucine Enkephalin.

Recently, Toth and co-workers have developed lipid-based drug-delivery systems for oral administration. The method involves attaching a lipoamino acid (LAA) or liposaccharide to the biologically active peptides. Lipoamino acids (LAA) combine the properties of amino acids (contains NH<sub>2</sub> and COOH functional groups) and lipids (hydrophobic side chains). They have successfully demonstrated this approach for various therapeutic peptides. This subject has been reviewed recently by Toth [72]. Morphine-6-glucuronide (M6G, Fig. 1) is a potent morphine metabolite that acts through  $\mu$  receptors. The metabolite M6G has been used clinically, [73] and has been reported to be more potent than morphine *in vivo* despite its lower affinity for  $\mu$  receptors, and to show reduced levels of respiratory depression [74]. These findings prompted Toth and co-workers to modify enkephalins at the C-terminal by attaching glucuronic acids by an amide linkage. These analogs (**11a—e**, Table 2) were synthesized by solid-phase strategy [75,76]. The attachment of one or two glucuronic acids to Leu-enkephalin increased activity by up to 40 fold, and increased selectivity towards  $\mu$ -opioid receptor. Some of the analogs showed appreciable stability and permeability when studied by Caco-2 assay as an *in vitro* model [77]. They also applied this strategy to enhance the bioavailability of somatostatin analogs [78].

Table 2. C-Terminal Glycosyl Enkephalin Amide Analogs

X =	Relative $\mu$ Potency	Relative Potency
<b>11a</b> 	0.97 ± 0.22	1.58 ± 0.10
<b>11b</b> 	2.74 ± 0.37	35.39 ± 5.45
<b>11c</b> 	0.14 ± 0.01	0.51 ± 0.03
<b>11d</b> 	0.52 ± 0.08	2.56 ± 0.17
<b>11e</b> 	0.012 ± 0.001	0.004 ± 0.0003

### GLYCOSYLATION OF ENKEPHALINS

Many proteins exist *in vivo* in glycosylated forms. It is known that glycosylated proteins have extended half-lives in serum. It is known that glycosylation greatly influences the biodistribution of glycoproteins, [79] including penetration of the BBB [80]. There is not a clear understanding of how glycosylation changes the physical chemistry of proteins, nor a complete catalog of the biochemical interactions of glycoproteins *in vivo* [81]. One strategy to produce systemically active enkephalin analogs is to produce glycosylated peptides that structurally mimic their larger glycoprotein cousins with the expectation that the biochemical behavior of the smaller glycopeptides will also demonstrate similar biodistribution properties. In fact, this seems to be true. The systematic study of the biodistribution of glycopeptide drugs may also lead to an increased understanding of glycoproteins. The smaller glycopeptides can be structurally altered by means of chemical synthesis to aid in these studies.

The synthesis of glycopeptides still poses significant synthetic challenges, particularly in large scale [82]. There are now several methods available to prepare N-linked or O-linked glycosylated amino acids. This subject has been reviewed recently [83,84]. Since the synthesis of natural N- or O-linked glycopeptides was very difficult in the past, the more easily synthesized neoglycopeptides (*e.g.* non-physiological glycoconjugates such as those in Table 3) have been more extensively explored.

### OPIOID NEO-GLYCOPEPTIDES

The first opioid neo-glycopeptides were synthesized by the Garcia-Anton group at the University of Salamanca, Spain. Their analogs had a  $\mu$ -selective Tyr-DMet-Gly-Phe-Pro- sequence with the C-terminal amide-linked carbohydrate portions consisting of -D-gluco- and -D-galactopyranose. These analogs showed as high as 2000 times the potency of morphine in the tail flick assay after *i.p.* administration [85]. The same group later introduced glucose

and galactose *via* O-linkage to the C-terminal of [D-Met<sup>2</sup>, Hyp<sup>3</sup>]enkephalin to improve its potency as well as selectivity. It was found that both glucose and galactose improve its antinociceptive activity, but there was a clear cut difference that the galactosyl analog was 57,000 times more potent than the glucosyl analog [86]. These analogs were the first O-linked glycopeptide analogs.

Horvat and co-workers also synthesized a series of neoglycopeptides based on either [Leu<sup>3</sup>]- or [Met<sup>5</sup>]enkephalin. The carbohydrate moieties were attached to the peptide by an ether-, ester-, amide- or N-alkyl- type linkage [87-90]. The GPI ( $\mu$ -receptor) and MVD ( $\delta$ -receptor) assays of these peptides revealed that bioactivity depended on the position, the identity of the sugar, and type of linkage. The  $\delta$ -anomer was about three times more active than the anomeric  $\alpha$ -glycoside. These peptides also showed modest antiviral activity against HIV-1. The authors concluded that the possible reason for antiviral activity could be due to the stability of the glycopeptides and/or a facilitated transport through cell membranes. Further investigation is needed before these glycopeptides could be considered as viable candidates for anti-HIV drugs [91]. There was no report on blood-brain barrier studies of these compounds.

### O-LINKED GLYCOPEPTIDES AND THE "AMPHIPATHIC HYPOTHESIS"

Work with these had to wait for effective synthetic methodology, which is exemplified by the work of Paulsen [92] and Jeanloz [93]. In the author's laboratory, several O-linked glycopeptide analogs of enkephalin were synthesized and studied [94]. The first series of glycopeptides produced, **12a–d**, (Fig. 6) was based on the  $\delta$ -selective DCDCE (D-Cys D-Cys Enkephalin) analog to improve bioavailability by attaching the hydrophilic sugar at the side chain of Ser. These disulfide bridged cyclic peptides penetrated the BBB when the glycosylated residue was at the C-terminus, but their potency was lost when the sugar was attached within the N-terminal message segment. Even though some analogs of the cyclic disulfide series crossed the BBB and produced

antinociception, [95] the presence of sulfur containing Cys amino acid made these compounds difficult to synthesize, along with a shorter shelf life. Thus, the focus turned to another  $\delta$ -selective opioid sequence, DTLES [96]. The analogs based on this sequence allowed for easier synthesis and purification on a much larger scale than the previous one, and in turn, allowed for more complete *in vivo* testing. The glycopeptides based on this pharmacophore are quite potent  $\delta$ -agonists, but still have appreciable  $\mu$ -activity. The analog containing  $\delta$ -D-Glc-L-Ser (**13d**) was compared with the non-glycosylated form (**13a**), and the cyclic disulfide (**12b**). Both **13d** and **13a** bind to the  $\delta$  receptor at concentrations with  $K_i$  values of  $\sim 2$  nM, and  $30$  nM at the  $\mu$  receptor. Additional studies have shown that the analgesic effects of **13d** are reversed by centrally administered naloxone methiodide [97]. Glycosylation of Ser<sup>6</sup> of **13d** led to a significant increase in enzymatic stability in both serum and brain, and increased BBB permeability. Antinociception studies showed that the glycosylated peptide gave significantly improved activity after *i.v.* administration, compared with the unglycosylated **13a** analog [98]. Based on these results, **13d** and **13f** appear to be ideal candidates for further development, perhaps even oral delivery [99].

These findings with glycopeptide **13d** encouraged the authors to explore the biological effects of additional analogs in which the parent pharmacophore was kept constant as the glycoside within the address segment was varied. These studies focused on altering the *amphipathicity* of the glycosylated enkephalins by increasing the number of attached carbohydrate residues (oligosaccharides) or the number of glycosylation sites (multiple monosaccharides), thereby increasing the hydrophilicity of the carbohydrate moieties. Amphipathicity was also changed by altering the orientation of the glycoside (D- & L-serine, and D- & L-threonine linkages) with respect to the lipophilic peptide moiety. Antinociception studies *in vivo* (*i.c.v.* and *i.v.*) were performed in addition to  $\mu/\delta$  binding studies and GPI/MVD functional assays. All of the glycosylated analogs in this series were 100-200 times more potent (20-100 pmol/mouse)

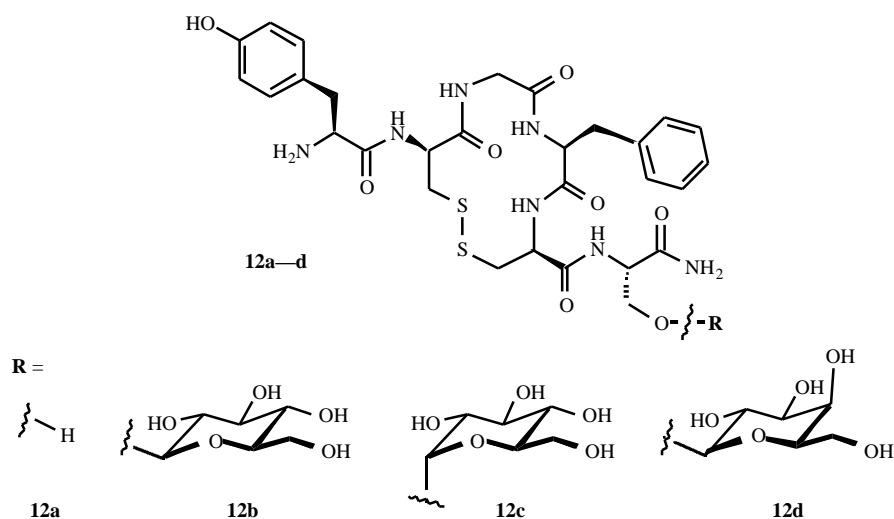


Fig. (6). Cyclic Disulfide Met-Enkephalins.

Table 3. *In Vitro* Binding and *In Vivo* Opioid Agonist Activity of Glycosyl DTLES Amides

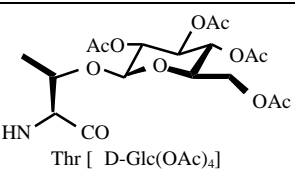
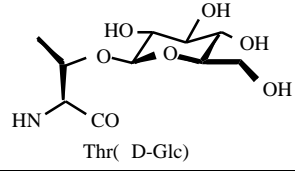
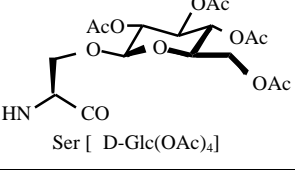
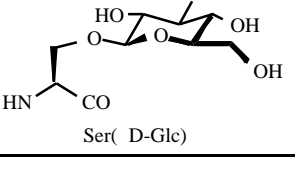
Drug	R =	(nM)	$\mu$ (nM)	MVD (nM)	GPI (nM)	A <sub>50</sub> i.c.v. (nmol)	A <sub>50</sub> i.v. ( $\mu$ mol/Kg)
13a		2.1	7.5	2.7	25	0.07	46.4
13b	 -D-Xyl	46.0	65.8	1.9	28.3	0.04	9.5
13c	 -D-Man	23.0	15.2	3.0	23.3	0.04	31.6
13d	 -D-Glc	2.4	7.6	1.6	34	0.02	11.4
13e	 -D-Glc-(1→4)-D-Glc	9.86	30.8	1.7	52.6	0.07	12.0
13f	 -D-Gal-(1→4)-D-Glc	17.3	40	5.72	34.8	0.02	3.2
13g	 -D-Gal-(1→6)-D-Glc	5.6*	36.6*	6.06	43.8	0.034	2.2
13h	 [ -D-Glc-(1→4)] <sub>2</sub> -D-Glc	46.3	98.3	7.7	71.7	0.06	10.9

\*Radioligand displacement data for **13g** was obtained from human DOR and MOR expressed in CHO cells. Other  $\mu$  binding data was obtained in the same way from homogenized mouse brain.

than morphine (2.7 nmol/mouse) when administered *i.c.v* [100]. Among them, the most potent (>200% the potency of morphine, molar basis) were the disaccharides **13f** (-lactoside), and **13g** (-melibioside). These analogs were more potent than the glucoside **13d**, and showed antinociceptive potencies greater than morphine following *i.v.* or *s.c.* administration. The addition of a third saccharide unit apparently reduced the systemic bioavailability. None of the bis-monosaccharides possessed the *in vivo* or *in vitro* potency of the analogs with a single glycosylation site. Taken together, this data is consistent with the hypothesis that the *amphipathicity* of the glycopeptide (not simply the hydrophilic or hydrophobic nature alone) greatly influences interaction with biological membranes, and that the resulting amphipathic behavior greatly influences both opioid binding and transport properties of the glycopeptides.

Additional support for this hypothesis was provided by NMR and CD studies with micelles and liposomes and a series of glycosylated enkephalins, **13a**, **13d**, **13e**, and **13h** [101]. As the degree of glycosylation increased (0–3 glucose units), membrane interaction (liposome binding) and transport rates (BBB uptake) correlated with amphipathicity, and not with hydrophilicity values. Two-dimensional NMR and Monte Carlo calculations showed that the hexa(glyco)peptides in this study adopted restricted conformations *at the surface* of micelles, and did not penetrate deep into their interior. This property, that is—the ability of the amphipathic glycopeptide to reside at the *interface* of a membrane-water boundary, is key to allowing for reversible interactions with membranes, and subsequent transcytosis across the BBB.

**Table 4.** *In Vitro* Binding & *In Vivo* Opioid Agonism of Glycosyl Deltorphin Amides

Drug	R =	(nM)	$\mu$ (nM)	MVD (nM)	GPI (nM)	A <sub>50</sub> <i>i.c.v.</i> (nmol)	A <sub>50</sub> <i>s.c.</i> ( $\mu$ mol/Kg)
<b>14a</b>	 Thr [ D-Glc(OAc) <sub>4</sub> ]	1.30±0.3	634.9±94	nd	nd	10.5 <sup>a</sup>	No activity for 150 $\mu$ M/kg dose
<b>14b</b>	 Thr( D-Glc)	1.55±0.5	578.9±38	nd	nd	11.3 <sup>a</sup>	96.4
<b>14c</b>	 Ser [ D-Glc(OAc) <sub>4</sub> ]	28±2.1	1915±210	2.7±0.4	1900±203	70 <sup>b</sup>	nd
<b>14d</b>	 Ser( D-Glc)	23±1.9	879±100	3.1±0.4	1600±133	44 <sup>b</sup>	nd

<sup>a</sup>tail-flick test <sup>b</sup>hot-plate test nd = not determined

## GLYCOSYLATION OF OTHER OPIOID PEPTIDES

The enkephalins are not the only antinociceptive message segments to provide the basis for glycopeptide drugs. Morphiceptin (H-Tyr-Pro-Phe-Pro-NH<sub>2</sub>) is derived from bovine  $\kappa$ -casein [102], and is very similar to human endogenous  $\mu$ -receptor agonists, namely endomorphin-1 (Tyr-Pro-Trp-Phe-NH<sub>2</sub>), endomorphin-2 (Tyr-Pro-Phe-Phe-NH<sub>2</sub>) [103]. This tetra-peptide provided a potent message segment for glycopeptides with  $\mu$ -receptor selectivity. Bardaji *et al.* have made analogs of morphiceptin by replacing Pro<sup>4</sup> with a hydroxyproline residue bearing -D-glucose and -D-galactose [104]. The resultant analogs showed an unexpected decrease in activity, especially the galactose derivative, which had no antinociceptive activity at all.

Exogenous peptides isolated from amphibian skin glands also carry highly potent opioid message segments, and have provided excellent candidates for transformation into glycopeptide analgesics. Specifically, the deltorphins (named for high delta specificity) and the dermorphins (named for dermal morphine-like substance) have also been identified as ligands for  $\delta$  and  $\mu$  opioid receptors, respectively. Dermorphin was found to be 500-1000 times more potent as an analgesic than morphine when administered *i.c.v.* in rats, but crossed the BBB more poorly than morphine [105]. In an effort to increase the serum half-life and BBB penetration, Tomatis and co-workers synthesized a series of deltorphin and dermorphin analogs (**14a–d**) in which a -D-glucose was attached to a Thr<sup>3</sup> or Thr<sup>7</sup> side chain [106] (Table 4). The binding studies and *in vivo* tail flick assay indicated that the Thr<sup>7</sup> glycosylated analog retained high receptor

Table 5. *In Vitro* Binding & *In Vivo* Opioid Agonism of Glycosyl Dermorphin Amides

Drug	R =	(nM)	$\mu$ (nM)	MVD (nM)	GPI (nM)	A <sub>50</sub> <i>i.c.v.</i> (nmol)	A <sub>50</sub> <i>s.c.</i> ( $\mu$ mol/Kg)
15a		86.8±14.7	0.46±0.06	nd	22.1±2.4	2.8 <sup>a</sup>	88.9
15b		67.0±22	0.29±0.02	nd	3.1±0.2	1.3 <sup>a</sup>	0.53
15c		1320±147	7.9±0.9	18±2.9	8.2±0.7	4.6 <sup>b</sup>	nd
15d		1250±150	2.4±0.3	40±5	3.5±0.4	1.4 <sup>b</sup>	nd

<sup>a</sup>tail-flick test <sup>b</sup>hot-plate test nd = not determined

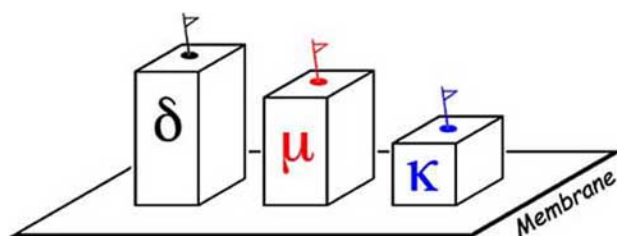


Fig. (7). Schwyzer's Membrane Compartment Hypothesis.

selectivity and remarkable *in vivo* activity. In addition, the glycosylated analog showed high blood to brain rate of influx, which they attributed to the glucose transporter GLUT-1. In contrast, the Thr<sup>4</sup> glycoside showed much less activity, which reinforces the notion that the message segment should not be altered if one wishes to retain opioid activity.

The Negri group in Italy prepared glycopeptide analogs (**15a–d**) of dermorphin [107] (Table 6). In these structures the carbohydrate moiety was attached through either a  $\beta$ -O-glycoside or a  $\beta$ -C-glycoside at the C-terminus. The resulting analogs, especially the acetylated glycosides (**15a** or **15c**), showed significant enzymatic stability. Examination of the acetylated glycoside in this system, as compared to the unprotected sugars, revealed some very interesting trends in the binding and antinociception. First, acetylation of glycoside was seen to hinder opioid activity of the compounds in the GPI assay ( $\mu$ -receptor), while it slightly enhanced activity in the MVD assay ( $\delta$ -receptor). This is contrary to what might be predicted by Schwyzer's *membrane compartment hypothesis*. This theory predicts that hydrophilic compounds will have a greater activity at the  $\delta$ -receptor (MVD assay) that is believed to reside in a more aqueous compartment than the  $\mu$ -site. When the acetyl-protected and un-acetylated analogs were compared *in vivo* by *s.c.* administration, they found that analogs containing the free sugars (*e.g.* **15b** and **15d**) possessed remarkable antinociceptive activity by peripheral injection, but that the more lipophilic acyl derivatives were much less active.

## MEMBRANE EFFECTS ON BINDING

In a classic study of peptide-membrane interaction, Merrifield and co-workers synthesized the enantio (all D amino acid), retro (sequence reversed), retro-enantio (sequence reversed and all D amino acid) isomers, as well as chimeric analogs of the bee venom melittin in order to evaluate its antibacterial, hemolytic and lipid bilayer activity. The results confirm the requirement for a specific cationic region located at the C-terminus, the amphipathic  $\alpha$ -helix, as well as the non-requirement of chirality—*i.e.* chirality plays no role in the activity. The authors suggest that these peptides do not function *via* any receptor or enzymatic process. The helix dipole is believed to play a vital role in orienting the peptides within the membrane. They suggest the following sequence for melittin-membrane interactions. The basic peptide monomer first binds to the lipid bilayer electrostatically, and then rearranges in such a way that the hydrophilic surface contacts polar head groups of the lipid and the hydrophobic residues insert into the apolar hydrocarbon chains of the fatty acids. This, in turn, is believed to induce an amphipathic helical conformation with a large dipole parallel to the axis of the helix. Finally, the peptides are believed to aggregate and rotate by interaction of the dipoles with the applied voltage to form ion conducting pores across the cell membrane [108].

Robert Schwyzer pointed out the importance of the membrane in peptide-receptor interactions with the development of his *membrane compartment theory*. According to Schwyzer, the “lipid phase of a cellular membrane will act as a matrix for the receptor.” [109]. Additionally, opioid receptor agonists are believed to have two segments, a *message* or recognition segment, and an *address* segment. It has been well established that the N-terminal sequence YGGF is a common message segment in opioid peptide, and is essential for the activity. An amphipathic helix may act as the address segment to guide the drug to a particular subset of receptors [110]. This address segment varies in the opioid peptides and is believed to be responsible for  $\mu/\delta$  selectivity. According to Schwyzer, the  $\delta$  receptor (binding site for enkephalins and

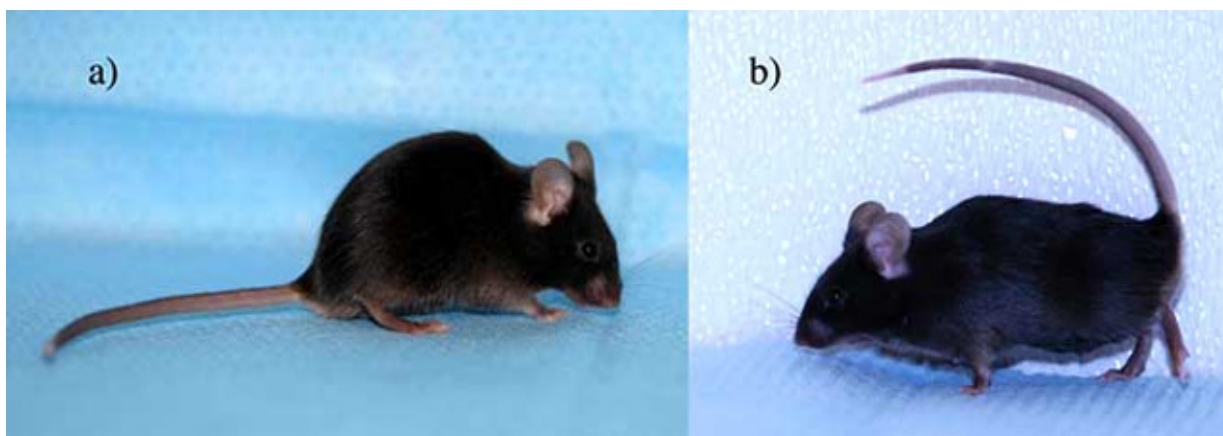


Fig. (8) Glycopeptide Antinociception vs Morphine Analgesia in the Mouse. Both mice have received equi-analgesic ( $A_{90}$ ) doses of drug, glycopeptide on the left (a), morphine on the right (b). Glycopeptide-induced analgesia does not cause increased locomotor activity, stereotypical circling, or Straub tail. Morphine-induced analgesia causes greatly increased locomotor activity, stereotypical circling and Straub tail. Photographs courtesy of Prof. Edward J. Bilsky, University of New England, Biddeford, Maine.

endorphins) projects further into the aqueous exterior of the cell, the  $\mu$  receptor (morphine binding site) resides in the anionic phospholipid head region and the receptor (dynorphin binding site) is buried within the lipid portion of the membrane.

### OUTLOOK FOR A GENERAL METHOD FOR GLYCOPEPTIDE TRANSPORT

This message-address theory of peptide-substrate interactions is not confined to opioid peptides, and has been demonstrated to be operative for antimicrobial peptides such as cecropins and magainins, as well as other systems. It is probably not accidental that the extremely potent conotoxins are heavily glycosylated. Studies on cobra toxin and melittin by Fletcher and co-workers have clearly exemplified the membrane compartmentalization and message-address dichotomy [111]. Thus, it would seem that glycosylation may be a general method for the introduction of surfactant properties into biologically active peptides, thereby increasing their ability to cross membrane barriers. Moreover, the  $\mu$ -selective nature of the enkephalin-based peptide ligand does not cause the same degree of narcotic side effects typical of  $\mu$ -selective opioids, such as morphine (Fig. 8).

### ACKNOWLEDGEMENT

We thank the Office of Naval Research (N00014-02-1-0471) and the National Science Foundation (CHE-9526909) for financial support.

### REFERENCES

- [1] (a) Kririkos, P.G.; Papadaki, S. *J. Am. Pharm. Assn.* **1968**, *8*, 446-447. (b) Wright, A.D. *Trans. Coll. Physicians Phila.* **1961**, *29* (July), 22-27. (c) *The History of Opium and Its Derivatives in Advances in Pain Research and Therapy*, Vol. 14, Benedetti, C., Ed.; Raven Press, New York, **1990**, Chapter 1, pp. 1-35, c) <http://opioids.com/timeline/>.
- [2] Brownstein, M.J. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 5391-5393.
- [3] Gutstein, H.B.; Akil, H. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10<sup>th</sup> Edition*, Hardman, J.G.; Limbird, L.E.; Gilman, A.G., Eds.; R.R. Donnelley and Sons, New York, **2001**, Chapter 23, pp. 569-620.
- [4] Hannon, J.P.; Bossone, C.A. *Am. J. Physiol.* **1991**, *261*, R1286-93.
- [5] Shavit, Y.; Epaulis, A.; Martin, R.C.; Terman, G.W.; Pechnick, R.N.; Zane, C.J.; Gale, R.P.; Liebeskind, J.C. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 7114-7. (b) Weber, R.J.; Pert, A. *Science* **1989**, *245*, 188-190. (c) Carr, D.J.J.; Serou, M. *Immunopharm.* **1995**, *31*, 59-71. (d) Gaveriaux, R.C.; Matthes, H.W.D.; Kieffer, B.L. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6326-6330. (e) Ni, X.; Gritman, K.R.; Eisenstein, T.K.; Adler, M.W.; Arfors, K.E.; Tuma, R.F. *Microvasc. Res.* **2000**, *60*, 121-30.
- [6] McNally, G.P.; Akil, H. In *Neuropsychopharmacology: the Fifth Generation of Progress*, Davis, K.L.; Charney, D.; Coyle, J.T.; Nemeroff, C. Eds, Lippincott Williams & Wilkins, Philadelphia, **2002**, Chapter 3, pp. 35-46.
- [7] Pert, C.A.; Snyder, S.H. *Science* **1973**, *179*, 1011-1014. (b) Simon, E.J.; Hiller, J.M.; Edelman, I. *Proc. Natl. Acad. Sci. USA.* **1973**, *70*, 1947-1951. (c) Terenius, L. *Acta Pharmacol Toxicol* **1973**, *32*, 317-320.
- [8] Kosterlitz, H.W.; Lord, J.A.H.; Paterson, S.J.; Waterfield, A.A. *Br. J. Pharmacol.* **1980**, *68*, 333-342.
- [9] Tseng, L.F. *Trends in Pharm. Sci.* **2001**, *22*, 623-630.
- [10] Sufuoglu, M.; Portoghese, P.S.; Takemori, A.E. *J. Pharmacol. Exp. Ther.* **1991**, *257*, 676-680. (b) Clark, J.A.; Liu, L.; Price, M.; Hersh, B.; Edelson, M.; Pasternak, G.W. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 461-468.
- [11] a) Kieffer, B.L.; Befort, K.; Gaveriaux-Ruff, C.; Hirth, C.G. *Proc. Natl. Acad. Sci.* **1992**, *89*, 12048-52. b) Evans, C.J.; Keith, D.E.; Morrison, H.; Magendzo, K.; Edwards, R.H. *Science* **1992**, *258*, 1952-55.
- [12] a) Fukuda, K.; Kato, S.; Mori, K.; Nishi, M.; Takeshima, H. *FEBS Lett.* **1993**, *327*, 311-14. b) Chen, Y.; Mesick, A.; Liu, J.; Hurley, J.A.; Yu, L. *Mol. Pharmacol.* **1993**, *44*, 8-12.
- [13] a) Yasuda, K.; Raynor, K.; Kong, H.; Breder, C.; Takeda, J.; Reisine, T.; Bell, G.I. *Proc. Natl. Acad. Sci.* **1993**, *90*, 6736-40. b) Minami, M.; Toya, T.; Kaaoy, Y.; Maekawa, K.; Nakamura, S.; Onogi, T. *FEBS Lett.* **1993**, *329*, 291-95.
- [14] Pasternak, G.W. *Neuroscientist* **2001**, *7*, 220-231.
- [15] Gaveriaux-Ruff, C.; Kieffer, B.L. *Neuropeptides* **2002**, *36*, 62-71.
- [16] Gomes, I.; Filipovska, J.; Jordan, B.A.; Devi, L.A. *Methods* **2002**, *27*, 358-365.
- [17] Chaturvedi, K.; Christoffers, K.H.; Singh, K.; Howells, R.D. *Biopolymers* **2000**, *55*, 334-346.
- [18] Hughes, J.; Smith, T.W.; Kosterlitz, H.W.; Fothergill, L.A.; Morgan, B.A.; Morris, H.R. *Nature (London)* **1975**, *258*, 577-579.
- [19] a) Janecka, A.; Fichna, J.; Janecki, T. *Curr. Top. Med. Chem.* **2004**, *4*, 1-17. b) Lipkowski, A.W.; Misicka, A.; Hruby, V.J.; Carr, D.B. *Pol. J. Chem.* **1994**, *68*, 907-912. c) Dooley Ct, Chung, N.N.; Wilkes, B.C.; Schiller, P.W.; Bidlack, J.M.; Pasternak, G.W.; Houghton R.A. *Science* **1994**, *266*, 2019-2022.
- [20] Horvat, S. *Curr. Med. Chem.: Central Nervous System Agents* **2001**, *1(2)*, 133-154.
- [21] Pardridge, W.M. *Brain Drug Targeting*, Cambridge University Press: Cambridge, **2001**.
- [22] Gutstein, H.B.; Akil, H. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10<sup>th</sup> Edition*, Hardman, J.G.; Limbird, L.E.; Gilman, A.G., Eds.; R.R. Donnelley and Sons, New York, **2001**, Chapter 23, pp. 569-619.
- [23] Höllt, V. *Annu. Rev. Pharmacol. Toxicol.* **1986**, *26*, 59-77.
- [24] Bunzow, J.R.; Saez, C.; Mortrud, M. *FEBS Lett.* **1994**, *347*, 279-282.
- [25] a) Meunier, J.C.; Mollereau, C.; Toll, L. *Nature* **1995**, *377*, 532-535. b) Pan, Y.X.; Xu, J.; Pasternak, G.W. *Biochem. J.* **1996**, *315*, 11-13.
- [26] Quirion, R.; Weiss, A.S. *Peptides* **1983**, *4*, 445-452.
- [27] Loh, H.H.; Tseng, L.F.; Wei, E.; Li, C.J. *Proc. Natl. Acad. Sci. USA* **1976**, *73*, 2895.
- [28] Zadina, J.E.; Hackler, L.; Ge, L.-J.; Kastin, A.J. *Nature* **1997**, *386*, 499-502.
- [29] Goldstein, A.; Tachibana, S.; Lowney, L.I.; Hunkapiller, M.; Hood, L. *Proc. Natl. Acad. Sci.* **1979**, *76*, 6666-6670.
- [30] Erspamer, V.; Melchiorri, P.; Falconeri-Erspamer, G.; Negri, L.; Corsi, R.; Severini, C.; Barra, D.; Simmaco, M.; Kriegl, G. *Proc. Natl. Acad. Sci.* **1989**, *86*, 5188-5192.
- [31] a) Brantl, V.; Teshemacher, H.; Henschel, A.; Lottspeich, F.; Hoppe-Seylers, Z. *Physiol. Chem.* **1979**, *360*, 1211-1216. b) Fukudome, S.; Yoshikawa, M. *FEBS Lett.* **1992**, *296*, 107-111. c) Fukudome, S.; Yoshikawa, M. *FEBS Lett.* **1993**, *316*, 17-19. d) Zadina, J.E.; Kastin, A.J.; Kersh, D.; Wyatt, A. *Life Sci.* **1992**, *51*, 869-869. e) Yunden, J.; Yoshikawa, M. *Peptides* **1999**, *20*, 957-962.
- [32] Yan, S.; Yunden, J.; Sonoda, S.; Doyama, N.; Lipkowski, A.W.; Kawamura, Y.; Yoshikawa, M. *FEBS Lett.* **2001**, *509*, 213-217.
- [33] Gentilucci, L. *Curr. Top. Med. Chem.* **2004**, *4*, 19-38.
- [34] Adessi, C.; Soto, C. *Current Med. Chem.* **2002**, *9*, 963-978.
- [35] Schiller, P.W.; Nguyen, T.M.-D.; Mazziak, L.; Lemieux C. *Biochem. Biophys. Res. Commun.* **1985**, *127*, 558-564.
- [36] Mosberg, H.I.; Hurst, R.; Hruby, V.J.; Gee, K.; Yamamura, H.I.; Galligan, J.J.; Burks, T.F. *Proc. Natl. Acad. Sci. USA.* **1983**, *80*, 5871-5874.
- [37] Svensson, C.I.; Rew, Y.; Malkmus, S.; Schiller, P.W.; Taulane, J.P.; Goodman, M.; Yaksh, L. *J. Pharm. Exp. Ther.* **2003**, *304*, 827-832.
- [38] (a) Hruby, V.J.; Gehrig, C.A. *Med. Res. Rev.* **1989**, *9*, 343-401. (b) Hruby, V.J.; Agnes, R.S. *Biopolymers* **1999**, *51*, 391-410. (c) Naqvi, T.; Haq, W.; Mathur, K.B. *Peptides* **1998**, *19*, 1277-1292.
- [39] (a) Handa, B.K.; Lane, A.C.; Lord, J.A.H.; Morgan, B.A.; Rance, M.J.; Smith, C.F.C. *Eur. J. Pharmacol.* **1981**, *70*, 531-540. (b) Gacel, G.; Dauge, V.; Breuzé, P.; Delay-Goyet, P.; Roques, B.P. *J. Med. Chem.* **1988**, *31*, 1891-1897. (c) Schiller, P.W.; Nguyen, T.M.-D.; Weltrowska, G.; Wilkes, B.C.; Marsden, B.J.; Lemieux, C.; Chung, N.N. *Proc. Natl. Acad. Sci. USA.* **1992**, *89*, 11871-11875. (d) Schiller, P.W.; Nguyen, T.M.-D.; Berezowska, I.;

- Dupuis, S.; Weltrowska, G.; Chung, N.N.; Lemieux, C. *Eur. J. Med. Chem.* **2000**, *35*, 895-901.
- [40] (a) Shimohigashi, Y.; Kamiya, H.; Costa, T.; Herz, A.; Stammer, C.H. *FEBS letters* **1988**, *233*, 289-293. (b) Shimohigashi, Y.; Costa, T.; Nitz, T.J.; Chen, H.C.; Stammer, C.H. *Biochem. Biophys. Res. Commun.* **1984**, *121*, 966-972.
- [41] Schiller, P.W.; Nguyen, T.M.D.; Weltrowska, G.; Wilkes, B.C.; Marsden, B.J.; Lemieux, C.; Chung, N.N. *Proc. Natl. Acad. Sci. USA.* **1992**, *89*, 11871-11875.
- [42] (a) Schiller, P.W.; Fundytus, M.E.; Merovitz, L.; Weltrowska, G.; Nguyen, T.M.D.; Lemieux, C.; Chung, N.N.; Coderre, T.J. *J. Med. Chem.* **1999**, *42*, 3520-3526. (b) Neilan, C.L.; Nguyen, T.M.D.; Schiller, P.W.; Pasternak, G.W. *Eur. J. Pharm.* **2001**, *419*, 15-23. (c) Bryant, S.D.; Jinsmaa, Y.; Salvadori, S.; Okada, Y.; Lazarus, L.H. *Biopolymers (Peptide Science)* **2003**, *71*, 86-102. (d) Hruby, V.J.; Balse, P.M. *Cur. Med. Chem.* **2000**, *7*, 945-970.
- [43] Adessi, C.; Soto, C. *Cur. Med. Chem.* **2002**, *9*, 963-978.
- [44] Pardridge, W.M. *Introduction to the Blood-Brain Barrier*, Cambridge University Press: New York, **1993**.
- [45] Crone, C. *Acta Physiol. Scand.* **1963**, *58*, 292-305.
- [46] Dohrmann, G.J. *Brain Res.* **1970**, *18*, 197-218.
- [47] (a) Pardridge, W.M. *Peptide Drug Delivery to the Brain*, Raven Press: New York, **1991**. (b) de Boer, A.G.; van der Sandt, I.C.J.; Gaillard, P.J. *Ann. Rev. Pharmacol. Tox.* **2003**, *43*, 629-656.
- [48] Breig, N.H.; Fredericks, W.R.; Holoway, H.W.; Soncrant, T.T.; Rapoport, S.I. *J. Pharmacol. Exp. Ther.* **1988**, *245*, 581-586.
- [49] (a) Coffey, R.J.; Burchiel, K. *Neurosurgery* **2002**, *50*(1), 78-86. (b) Dahl, J.B.; Jeppesen, I.S.; Jorgensen, H.; Wetterslev, J.; Moiniche, S. *Anesthesiology* **1999**, *91*(6), 1919-1927.
- [50] Egleton, R.D.; Davis, T.P. *Peptides* **1997**, *18*, 1431-1439.
- [51] Vannucci, S.J.; Maher, F.; Simpson, I.A. *Glia*, **1997**, *21*, 2-21.
- [52] Oldendorf, W.H.; Szabo, J. *Am. J. Physiol.* **1976**, *230*, 94-98.
- [53] Maresh, G.A.; Kastin, A.J.; Brown, T.T.; Zadina, J.E.; Banks, W.A. *Brain Res.* **1999**, *839*, 336-340.
- [54] Ermisch, A.; Brust, P.; Kretzschmar, R.; Rühle, H. *Physiol. Rev.* **1993**, *73*, 489-527.
- [55] (a) Rapoport, S.I.; Klee, W.A.; Pettigrew, K.D.; Ohno, K. *Science* **1980**, *207*, 84-86. (b) Banks, W.A.; Kastin, A.J.; Fischman, A.J.; Coy, D.H.; Strauss, S.L. *Am. J. Physiol.* **1986**, *251*, E477-E482. (c) Hauser, M.; Donhardt, A.M.; Barnes, D.; Naider, F.; Becker, J.M. *J. Biol. Chem.* **2000**, *275*, 3037-3041.
- [56] Egleton, R.D.; Abbruscato, T.J.; Thomas, S.A.; Davis, T.P. *J. Pharm. Sci.* **1998**, *87*, 1433-1439.
- [57] Terasaki, T.; Hirai, K.; Sato, H.; Kang, Y.-S.; Tsuji, A. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 351-357.
- [58] Deguchi, Y.; Miyakawa, Y.; Sakurada, S.; Naito, Y.; Motimoto, K.; Ohtsuki, S.; Hosoya, K.; Terasaki, T. *J. Neurochem.* **2003**, *84*, 1154-1161.
- [59] (a) Tamai, I.; Sai, Y.; Kobayashi, H.; Kamata, M.; Wakamiya, T.; Tsuji, A. *J. Pharmacol. Exp. Ther.* **1997**, *280*, 410-415. (b) Wakamiya, T.; Kamata, M.; Kusumoto, S.; Kobayashi, H.; Sai, Y.; Tamai, I.; Tsuji, A. *Bull. Chem. Soc. Jpn.* **1998**, *78*, 699-709. (c) Tsuji, A.; Tamai, I. *Adv. Drug Delivery Rev.* **1999**, *36*, 277-290.
- [60] Tamsamani, J.; Rousselle, C.; Rees, A.R.; Scherrmann, J.-M. Vector-mediated drug delivery to the brain. *Expert Opinion on Biological Therapy* **2001**, *1*(5), 773-782.
- [61] Pardridge, W.M. *Adv. Drug Delivery Rev.* **1995**, *15*, 109-146.
- [62] Bodor, N.; Prokai, L.; Wu, W.M.; Farag, H.; Jonalagadda, S.; Kawamura, M.; Simpkins, J. *Science* **1992**, *257*, 1698-1700.
- [63] (a) Wang, b.; Nimkar, K.; wang, W.; Zhang, H.; Shan, D.; Gudmundsson, O.; Gangwar, S.; Siahaan, T.; Borchardt, R.T. *J. Pep. Res.* **1999**, *53*, 370-382. (b) Bak, A.; Gudmundsson, O.; Friis, G.J.; Siahaan, T.; Borchardt, R.T. *J. Pep. Res.* **1999**, *53*, 393-402. (c) Ouyang, H.; Borchardt, R.T.; Siahaan, T.J.; Vander Velde, D. *J. Pep. Res.* **2002**, *59*, 183-195.
- [64] (a) Yang, J.Z.; Chen, W.; Borchardt, R.T. *J. Pharmacol. Exp. Ther.* **2002**, *303*, 840-848. (b) Chen, W.; Yang, J.Z.; Anderson, R.; Nielsen, L.H.; Borchardt, R.T. *J. Pharmacol. Exp. Ther.* **2002**, *303*, 849-857.
- [65] Fredholt, K.; Adrian, C.; Just, L.; Larsen, D.H.; Weng, S.; Moss, B.; Friis, G.J. *J. Control. Rel.* **2000**, *63*, 261-273.
- [66] Schroeder, U.; Sommerfeld, P.; Ulrich, S.; Sabel, B.A. *J. Pharm. Sci.* **1998**, *87*, 1305-1307.
- [67] Alyautdin, R.; Gothier, V.; Petrov, V.; Kharkevich, D.; Kreuter, J.; *Eur. J. Pharm. Biopharm.* **1995**, *41*, 44-48.
- [68] Schroeder, U.; Sommerfeld, P.; Sabel, B.A. *Peptides* **1998**, *19*, 777-780.
- [69] Olivier, J.C.; Fenart, L.; Chauvet, R.; Pariat, C.; Cecchelli, R.; Couet, W. *Pharm. Res.* **1999**, *16*, 1836-1842.
- [70] Kreuter, J.; Ramage, P.; Petrov, V.; Hamm, S.; Gelperina, S.; Engelhardt, B.; Alyautdin, R.; Briesen, H.; Begley, D. *Pharm. Res.* **2003**, *20*, 409-416.
- [71] Tsuzuki, T.; Hama, T.; Hibi, T.; Konishi, R.; Futaki, S.; Kitagawa, K. *Biochem. Pharmacol.* **1991**, *41*, R5-R8.
- [72] Wong, A.; Toth, I.; *Curr. Med. Chem.* **2001**, *8*, 1123-1136.
- [73] Andersen, G.; Chistrup, L.; Sjøgren, P. *J. Pain Symp. Management*, **2003**, *25*, 74-91.
- [74] Smith, M.T.; South, S.M. *Pain Rev.* **2001**, *8*, 171-191.
- [75] Drouillat, B.; Kellam, B.; Dekany, G.; Starr, M.S.; Toth, I. *Bioorg. Med. Chem. Lett.*, **1997**, *7*, 2247-2250.
- [76] Kellam, B.; Drouillat, B.; Dekany, G.; Starr, M.S.; Toth, I. *Int. J. Pharm.* **1998**, *161*, 55-64.
- [77] Wong, A.K.; Ross, B.P.; Chan, Y.N.; Artursson, P.; Lazorova, L.; Jones, A.; Toth, I.; *Eur. J. Pharm. Sci.* **2002**, *16*, 113-118.
- [78] Toth, I.; Malkinson, J.P.; Flinn, N.S.; Drouillat, B.; Horvath, A. *J. Med. Chem.* **1999**, *42*, 4010-4013.
- [79] Lis, H.; Sharon, N. *Eur. J. Biochem.* **1993**, *3*, 97-130.
- [80] Poduslo, J.F.; Curran, G.L. *Molecular Brain Research* **1994**, *23*, 157-162.
- [81] (a) O'Connors, S.E.; Imperiali, B. *Chem. Biol.* **1996**, *3*, 803-812. (b) Dwek, R.A. *Chem. Rev.* **1996**, *96*, 683-720. (c) Varki, A. *Glycobiology* **1993**, *3*, 97-130. (d) Kobata, A. *Eur. J. Biochem.* **1992**, *209*, 483-501.
- [82] Burger, K.; Kluge, M.; Fehn, S.; Koksche, B.; Hennig, L.; Müller, G. *Angew. Chem. Int. Ed.* **1999**, *38*, 1414-1416.
- [83] Seitz, O. *Chem. Biochem.* **2000**, *1*, 214-246.
- [84] Herzner, H.; Reipen, T.; Schultz, M.; Kunz, H. *Chem. Rev.* **2000**, *100*, 4495-4537.
- [85] Torres, J.L.; Reig, F.; Valencia, G.; Rodrguez, R.; Garcia-Anton, J.M. *Int. J. Pept. Prot. Res.* **1988**, *31*, 474-480.
- [86] Rodriguez, R.E.; Rodriguez, F.D.; Sacristán, M.P.; Torres, J.L.; Valencia, G.; Garcia Anton, J.M. *Neurosci. Lett.* **1989**, *101*, 89-94.
- [87] Varga, L.; Horvat, S.; Lemieux, C.; Schiller, P.W. *Int. J. Peptide Protein Res.* **1987**, *30*, 371-378.
- [88] Horvat, J.; Horvat, S.; Lemieux, C.; Schiller, P.W. *Int. J. Peptide Protein Res.* **1988**, *31*, 499-507.
- [89] Horvat, S.; Horvat, J.; Varga-Defterdarovic, L.; Pavelic, K.; Chung, N.N.; Schiller, P.W. *Int. J. Peptide Protein Res.* **1993**, *41*, 309-404.
- [90] Skuric, M.; Horvat, J.; Horvat, S.; Chung, N.N.; Schiller, P.W. *Int. J. Peptide Protein Res.* **1994**, *43*, 402-409.
- [91] Horvat, S.; Varga, L.; Pfutzner, A.; Suhartono, H.; Rubsamen-Waigmann, H. *Helv. Chim. Acta.* **1991**, *74*, 951-955.
- [92] Paulsen, H. *Angew. Chem. Int. Ed. Engl.* **1982**, *21*, 155-173.
- [93] Garg, H.G.; Jeanloz, R.W. *Adv. Carbohydr. Chem. Biochem.* **1985**, *43*, 135-201.
- [94] Mitchell, S.A.; Pratt, M.R.; Hruby, V. J.; Polt, R. *J. Org. Chem.* **2001**, *66*, 2327-2342.
- [95] Polt, R.; Porreca, F.; Szabo, L.Z.; Bilsky, E.J.; Davis, P.; Abbruscato, T.J.; Davis, T.P.; Horvath, R.; Yamamura, H.I.; Hruby, V.J. *Proc. Natl. Acad. Sci.* **1994**, *91*, 7114-8.
- [96] Zajac, J.M.; Gacel, G.; Petit, F.; Dodey, P.; Rossignol, P.; Rogues, B.P. *Biochem. Biophys. Res. Commun.* **1983**, *111*, 390-397.
- [97] Egleton, R.D.; Mitchell, S.A.; Huber, J.D.; Janders, J.; Stropova, D.; Polt, R.; Yamamura, H.I.; Hruby, V.J.; Davis, T.P. *Brain Res.* **2000**, *881*, 37-46.
- [98] Egleton, R.D.; Mitchell, S.A.; Huber, J.D.; Palian, M.M.; Polt, R.; Davis, T.P. *J. Pharmacol. Exp. Ther.* **2001**, *299*, 967-972.
- [99] Bilsky, E.J.; Egleton, R.D.; Mitchell, S.A.; Palian, M.M.; Davis, P.; Huber, J.D.; Jones, H.; Yamamura, H.I.; Janders, J.; Davis, T.P.; Porreca, F.; Hruby, V.J.; Polt, R. *J. Med. Chem.* **2000**, *43*, 2586-2590.
- [100] Elmagbari, N.O.; Egleton, R.D.; Palian, M.M.; Lowery, J.J.; Schmid, W.R.; Davis, P.; Navratilova, E.; Dhanasekaran, M.; Keyari, C.M.; Yamamura, H.I.; Porreca, F.; Hruby, V.J.; Polt, R.; Bilsky, E.J. *J. Pharm. Exp. Therap.* **2004**, in press.
- [101] Palian, M.M.; Boguslavsky, V.I.; O'Brien, D.F.; Polt, R. *J. Am. Chem. Soc.* **2003**, *125*, 5823-5831.
- [102] (a) Brantl, V.; Teschemacher, H.; Henschen, A.; Lottspeich, F. *Hoppe-Seyler's Z. Physiol. Chem.* **1979**, *360*, 1211. (b) Zioudrou, C.; Streaty, R.A.; Klee, W.A. *J. Biol. Chem.*, **1979**, *254*, 2446.

- [103] Elmagbari, N.O.; Egleton, R.D.; Palian, M.M.; Lowery, J.J.; Schmid, W.R.; Davis, P.; Navratilova, E.; Dhanasekaran, M.; Keyari, C.M.; Yamamura, H.I.; Porreca, F.; Hruby, V.J.; Polt, R.; Bilsky, E.J. *J. Pharm. Exp. Therap.* **2004**, in press.
- [104] Bardaji, E.; Torres, J.L.; Clapes, P.; Aalbericio, F.; Barany, G.; Rodrigues, R.E.; Sacristan, M.P.; Valencia, G. *J. Chem. Soc. Perkin Trans.*, **1991**, *1*, 1755-9.
- [105] Fiori, A.; Cardelli, P.; Negri, L.; Savi, M.R.; Storm, R. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 9469-9474.
- [106] Tomatis, R.; Marastoni, M.; Balboni, G.; Guerrini, R.; Capasso, A.; Sorrentino, L.; Santagada, V.; Caliendo, G.; Lazarus, L.H.; Salvadori, S. *J. Med. Chem.* **1997**, *40*, 2948-52.
- [107] Negri, L.; Lattanzi, R.; Tabacco, F.; Orru, L.; Severini, C.; Scolaro, B.; Rocchi, R. *J. Med. Chem.* **1999**, *42*, 400-404.
- [108] Juvadi, P.; Vunnam, S.; Merrifield, R.B. *J. Am. Chem. Soc.* **1996**, *118*, 8969-8997.
- [109] Schwyzer, R.; Gysen, B. *Arch. Biochem. Biophys.* **1983**, *225*, 467-474.
- [110] Schwyzer, R. *Biochemistry* **1986**, *25*, 6336-6342.
- [111] Fletcher, J.E.; Jiang, M.S. *Toxicon*, **1993**, *31*, 669-95.

---

Received: June 07, 2004

Accepted: July 31, 2004